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CARRIER FOR ABSORBING BIOLOGICALLY ACTIVE SUBSTANCES
[Seiri kassei busshitsu kyuchaku yo tantai]

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[Claims] /2*

[Claim 1] A carrier for absorbing a biologically active substance comprising fine particles of hydroxyapatite with an average particle diameter of 500 nm or less surface-processed with albumin and/or a polyhydric organic acid.

[Claim 2] A medicinal preparation absorbing a biologically active substance containing fine particles of hydroxyapatite with an average particle diameter of 500 nm or less surface-processed with albumin and/or a polyhydric organic acid.

[Claim 3] The medicinal preparation described in Claim 2 also containing sugars or amino acids.

[Claim 4] A method for stabilizing a hydroxyapatite fine particle suspension, wherein fine particles of hydroxyapatite with an average particle diameter of 500 nm or less are surface-processed with albumin and/or a polyhydric organic acid.

[Claim 5] The stabilization method described in Claim 4, wherein sugars or amino acids are also added.

[Claim 6] The stabilization method described in Claim 4 or Claim 5, wherein the fine particles of hydroxyapatite absorb a biologically active substance.

[Detailed Description of the Invention]

[0001]

[Field of Industrial Application] The present invention relates to a carrier for absorbing a biologically active substance and a

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* Numbers in the margin indicate pagination in the foreign text.

medicinal preparation in which a biologically active substance is absorbed by this carrier.

[0002]

[Prior Art] In the treatment of different diseases, it is critical that the required amount of medicine reach the appropriate sites in order for the efficacy and safety of the medicine to be satisfied. Various methods have been proposed to control the release of medicines and determine their target orientation. One means of absorbing a medicine in a carrier with high biological affinity and gradually release the medicine is known as the drug delivery system (DDS).

[0003] Hydroxyapatite, which is the same as the component in bones and teeth, is well known as a medical material because of its high biological affinity. Recently, attempts have been made to absorb an anti-tumor drug in 30-50 μm particles of hydroxyapatite and administer this preparation.

[0004]

[Problem Solved by the Invention] However, the 30-50 μm diameter of the hydroxyapatite particles used as the carrier for the anti-tumor drug is large, and occludes fine capillary vessels when administered to humans. As a result, intravascular administration is not possible. Making the hydroxyapatite finer has been considered in order to overcome this problem. However, while making hydroxyapatite finer prevents occlusion of capillary vessels, the finer particles are much more likely to aggregate, adversely affecting dispersion stability. In

other words, these particles cannot be used as a medicinal carrier. Accordingly, the purpose of the present invention is to provide a carrier for absorbing a biologically active substance and a medicinal preparation using this carrier which has good biological affinity and which can be used in intravascular administration.

[0005]

[Means of Solving the Problem] The present inventors conducted extensive research on the relationship between fine particles of hydroxyapatite and dispersion stability, on the absorptivity of biologically active substances in these fine particles, and on the stability and efficacy of fine particles of hydroxyapatite. As a result, they found that fine particles of hydroxyapatite with an average particle diameter of 500 nm or less did not aggregate and had good dispersion stability if the surface of these fine particles was treated with albumin and/or a polyhydric organic acid, that these fine particles could be used in intravenous administration because of their small particle size, and that these surface-treated fine particles of hydroxyapatite having absorbed a biologically active substance had good efficacy and stability. The present invention is a product of these findings.

[0006] In other words, the present invention is a carrier for absorbing a biologically active substance comprising fine particles of hydroxyapatite with an average particle diameter of 500 nm or less surface-processed with albumin and/or a polyhydric organic acid.

[0007] The present invention is also a medicinal preparation absorbing a biologically active substance containing fine particles of hydroxyapatite with an average particle diameter of 500 nm or less surface-processed with albumin and/or a polyhydric organic acid.

[0008] In addition, the present invention is a method for stabilizing a hydroxyapatite fine particle suspension, wherein fine particles of hydroxyapatite with an average particle diameter of 500 nm or less are surface-processed with albumin and/or a polyhydric organic acid.

[0009] The fine particles of hydroxyapatite (HA) used as the carrier for a biologically active substance have an average particle diameter of 500 nm or less. If the average particle diameter exceeds 500 nm, blood vessels are occluded after intravascular administration. Preferably, the average particle diameter of the HA fine particles is 100 nm or less. These HA fine particles can be adjusted, for example, by subjecting large diameter HA particles to ultrasound or a nanomizer processing. Making the particles finer using nanomizer processing is preferred.

[0010] Human serum albumin is preferred as the albumin used to surface-process the HA fine particles. The polyhydric organic acid can be citric acid, polylactic acid or polyglutamic acid. Among these, citric acid is preferred.

[0011] The HA fine particles can be surface-processed while the HA fine particles are being made finer, after the particles have been made finer, or after the biologically active substance has been

absorbed. There are no particular restrictions on the surface-processing means. The HA fine particles can be immersed in a solution containing albumin and/or a polyhydric organic acid, stirred after immersion, or subjected to nanomizing or ultrasound processing after immersion. There are no restrictions on the amount of albumin and/or polyhydric organic acid used here. However, in the case of albumin, 1/20 or more can be used with respect t the HA fine particles in /3 terms of weight ratio.

[0012] The carrier for absorbing a biologically active substance obtained in this manner does not experience aggregation in the aqueous suspension even when stored for a long period of time and the dispersion stability is good. When a stabilizer such as a sugar or amino acid is added to the aqueous suspension, the stability is even better. Preferred examples of sugars used as stabilizers include monosaccharides, disaccharides and sugar alcohols such as mannose, galactose and sucrose. The amino acids include arginine and glycine. There are no particular restrictions on the stabilizer concentration, but 0.0001 to 20 wt% is preferred.

[0013] There are no particular restrictions on the biologically active substances that can be absorbed by the carrier in the present invention. Examples include the pharmacologically active components of different medicines such as biologically active peptides, anti-tumor agents, antibodies, anti-HIV agents, anti-rheumatics, analgesics, and various immunogenic substances. Bioassay substances (kits) for absorbing antigens, etc. can also be used.

[0014] There are no particular restrictions on the method used to absorb biologically active substances with the carrier. Chemical absorption can be performed, for example, via a cross-linking agent such as calcium-bonded proteins. The biologically active substance can be absorbed by the carrier before or after the surface of the HA fine particles is treated. If the surface treatment is performed using albumin, the substance should be absorbed before the surface treatment. In the case of surface treatment with a polyhydric organic acid, the absorption can be performed before or after the surface treatment.

[0015] The HA fine particles with an absorbed biologically active substance do not aggregate, have good dispersion stability, and gradually release the biologically active substance. This makes it easy to control the serum concentration of the biologically active substance, and provides a medicine with good efficacy and stability. This medicinal preparation does not occlude capillary blood vessels even when administered intravenously, and is especially appropriate for injection.

[0016]

[Working Examples] The following is a more detailed explanation of the present invention with reference to the working examples. The present invention is by no means limited to these working examples.

[0017] Working Example 1

(1) Using human serum albumin (HSA) as the surface treatment protein and low molecular weight gelatin, the final concentrations

were 0.5 mg/ml, 1.5 mg/ml, and 4.5 mg/ml (all aqueous solutions). The particles were made finer by adding a surface treatment agent to HA (Central Glass, final concentration 1 mg/ml) suspended in water, and then performing the nanomizer process (Nanomizer, Ltd.) five times at room temperature and at 1000 kg/cm^2.

[0018] The dispersion of the surface-treated HA fine particles was measured using a granularity distribution measuring device (Shimazu Works), and the average particle diameter was determined. The effect of the different surface-treating agents on the dispersion is shown in Table 1.

[0019]

[Table 1]

	HSA			Gelatin		
	0.5 mg/ml	1.5 mg/ml	4.5 mg/ml	0.5 mg/ml	1.5 mg/ml	4.5 mg/ml
Avg. Particle Dia. (μm)	0.06	0.07	0.06	0.78	0.96	0.90

[0020] From Table 1, it is clear that the average particle diameter of the added HSA was 100 nm or less, and that there was no aggregation and good dispersion properties no matter how much was added. When gelatin was added, aggregation occurred and approximately 80% had an average particle diameter of 500 nm or more no matter how much was added.

[0021] Working Example 2

Ultrasound processing was performed for one minute on the HA aqueous suspension at a final HA concentration of 10 mg/ml, HSA was added, ultrasound treatment was performed for another minute, and the

final HSA concentration required for dispersion and stabilization was studied. The results are shown in Table 2.

[0022]

[Table 2]

HSA Concentration (mg/ml)	Avg. Particle Dia. (nm)
0	3655
0.25	275
0.5	68
1	66
2	67
4	73
8	68

[0023] It is clear from Table 2 that a 1:20 weight ratio of HSA to HA provides a sufficient dispersion and stabilization effect.

[0024] Working Example 3

HA (final concentration 7.4 mg/ml) was nanomized five times at room temperature and at 1000 kg/cm^2 in media containing various acids, and the dispersion properties were studied. The media were water (not pH adjusted), nitric acid (diluted nitric acid with a pH of 4.5), acetic acid (adjusted to a pH of 4.5 using NaOH), succinic acid (adjusted to a pH of 4.5 using NaOH) and citric acid (adjusted to a pH of 4.5 using NaOH). The results are shown in Table 3. /4

[0025]

[Table 3]

Medium	Avg. Particle Diameter (nm)	
	pH 4	pH 5
Water (Not pH Adjusted)	(3569)	
Nitric Acid	4591	4508
Acetic Acid	4585	4486
Succinic Acid	4906	3485
Citric Acid	244	50

[0026] From Table 3, it is clear that inorganic acids and monohydric organic acids do not have an HA dispersion and stabilization effect. However, a polyhydric acid such as citric acid clearly has a superior HA dispersion and stabilization effect.

[0027] Working Example 4

The storage stability of HSA surface-treated HA fine particle dispersions was studied. An HA aqueous suspension (final concentration 1 mg/ml) was nanomized five times in the presence of 0.5 to 4.5 mg/ml HSA at room temperature and at 1000 kg/cm^2. The storage stability of the resulting HA dispersions was examined after storage for three weeks at 4°C. The effect of various additives was compared at the same time. The additives (final concentration 0.9%) were mannose (MAN), galactose (GAL), sucrose (SUC), arginine (ARG) and glycine (GLY). The results are shown in Table 4. The numbers in Table 4 denote the average particle diameters (μm).

[0028]

[Table 4]

Dispersion Liquid Storage Stability (4°C, 3 Weeks)

HSA (mg/ml) \ Additive	-	MAN	GAL	SUC	ARG	GLY
0.5	0.06	0.07	0.07	0.07	0.35	0.12
1.5	0.07	0.06	0.06	0.06	0.07	0.07
4.5	0.06	0.06	0.06	0.06	0.06	0.07

[0029] Working Example 5

The stability of the HA dispersion obtained in Working Example 4 was measured after being freeze-dried. The results are shown in Table 5. The numbers in Table 5 denote the average particle diameters (μm)

[0030]

[Table 5]

Stability After Freeze Drying (2 Days After Drying)

HSA (mg/ml) \ Additive	-	MAN	GAL	SUC	ARG	GLY
0.5	0.59	0.07	0.07	0.07	0.16	0.63
1.5	0.14	0.06	0.06	0.06	0.07	0.07
4.5	0.07	0.07	0.06	0.07	0.07	0.07

[0031] From Table 5, it is clear that the surface-treated HA fine particles in the present invention are stable even after being freeze-dried. However, aggregation was observed when the concentration of surface treatment agent is low. Here, however, the aggregation can be suppressed by adding a stabilizer.

[0032] Working Example 6

100 μl of peroxidase-bonded antibodies (peroxidase-bonded mouse monoclonal antibodies for measuring HBs antibodies, Abbot Japan) was added to 20 mg/0.5 ml HA, and ultrasound treatment was performed for 20 seconds. Afterwards, absorption was performed for five hours at 4°C, 1.5 mg/ml of HSA was added, and ultrasound processing was performed again for 20 seconds. Next, the HA fine particles were removed using centrifugation. This HA was rinsed five times in water and resuspended in water. This suspension was then divided in two, with some serving as the enzyme-reaction substance and some being centrifuged to obtain a supernatant. The peroxidase substrate was added to the substance and supernatant, and an enzyme reaction performed. As a result, an enzyme reaction was clearly observed in the substance, but no enzyme reaction was observed at all in the

supernatant. Therefore, the peroxidase-bonded mouse monoclonal antibodies were clearly absorbed by the HA itself maintaining this activity, and no active substance became free once absorbed. /5

[0033] Working Example 7

HA was suspended (10 mg/ml) in a citric acid buffer solution, and this was nanomized five times at 1000 kg/cm^2. After rinsing the resulting HA fine particles in water, they were resuspended in water. Enzyme-derived RNA was added and absorbed at 4°C overnight, and the amount of RNA (OD260) in the supernatant was measured. From the results shown in Table 6, it is clear that the amount absorbed was suitable for the absorbance difference in group 2 and group 3, and the RNA was sufficiently absorbed.

[0034]

[Table 6]

System	OD
Group 1 HA 10 mg/ml, No RNA	0.012
Group 2 HA 10 mg/ml, RNA	0.181
Group 3 No HA, RNA	0.797

[0035] Working Example 8

HA with the following particle diameter distributions was prepared and were compared for stability.

(1) Sample N with the particle diameter distribution in Table 7 and Sample P with the particle diameter distribution in Table 8 were used. In Sample N, 1.5 mg/ml HSA was added to 7.4 mg/ml HA (final concentration), and the mixture was nanomized five times at 1000 kg/cm^2. In Sample P, 2 mg/ml HSA (aqueous solution) was added to 10.6

mg/ml HA (final concentration), and the mixture was processed for nine minutes in a Polytron (Kinematica, Switzerland).

[0036]

[Table 7]

Sample N Particle Diameter Distribution

Particle Diameter (nm)	Relative Percentage (%)
150-100	1.1
100-80	8.2
80-60	44.1
60-50	30.2
< 50	16.5

[0037]

[Table 8]

Sample N Particle Diameter Distribution

Particle Diameter (nm)	Relative Percentage (%)
3000-2000	0.6
2000-1500	1.3
1500-1000	4.9
1000-800	10.6
800-600	28.4
600-500	15.6
500-400	13.0
400-300	9.2
< 300	16.4

[0038] (2) Animals: Groups of five 6 week-old female ddY mice were used.

[0039] (3) Group Names and Amounts Administered (mgHA/mouse): See Table 9.

[0040]

[Table 9]

Group Name	Sample N	Group Name	Sample P
N-1	0.74	P-1	1.48
N-2	1.48	P-2	2.11
N-3	3.70	P-3	2.64
N-4	5.18	P-4	3.70
N-5	7.40		

[0041] (4) Administration Method: Administered once via caudal intravenous injection.

[0042] (5) No. of Surviving Mice One Week After Administration: See Table 10. Nearly all of the surviving mice behaved like normal mice.

[0043]

[Table 10]

Group Name	Dead Mice/All Mice (Day 7)	Group Name	Dead Mice/All Mice (Day 7)
N-1	0/5	P-1	0/5
N-2	0/5	P-2	0/5
N-3	0/5	P-3	5/5
N-4	0/5	P-4	5/5
N-5	0/5		

[0044] From these result, it is clear that the surface /6 processing performed in the present invention improves stability. In the case of Sample N, all of the mice survived even when 7.4 mg was administered intravenously, and the LD_{50} was 250 mg/kg. In the case of Sample P, the LD_{50} was 70-90 mg/kg. Death occurred immediately after administration, and the cause was clearly vascular occlusion. The stability of the dispersion-stabilized fine particle carrier of the present invention was high.

[0045] Working Example 9

After administering the rat antibodies for mouse CD4 and CD8 to the mice, the peripheral blood lymphocytes, the lymph nodes and spleens were removed. The drug targeting was studied by determining

the lymphocyte subpopulation percentages in these organs. The following antibodies (and control) were used.

Rat Antibodies: Anti-Mouse CD4 Antibody, Anti-Mouse CD8 Antibody

Rat Serum Ig

[0046] (a) HA-Absorbed Antibody Preparation

The antibodies (antibody final concentration 1 mg/ml) was added to an HA aqueous suspension (final concentration 5 mg/ml), and nanomizing was performed in the same manner as the first working example. After allowing absorption to occur overnight at 4°C, the HA was retrieved using centrifugation and the unabsorbed substance was removed. After HSA (0.5 mg/ml) was added, dispersion processing was performed to obtain a sample. The amount of antibodies in the supernatant was measured to grasp the amount of absorbed antibodies. The following are the absorption rates.

Anti-Mouse CD4 Antibody: 75.4%

Anti-Mouse CD8 Antibody: 63.8%

Rat Serum Ig: 66.0%

(b) Mice: C57BL/6 Mice (6 Weeks Old, Female, 3 per Group)

(c) Administration: 0.1 ml was administered twice, back-to-back, to the veins at the back of the eye.

Total Amount Administered:

Anti-Mouse CD4 Antibody: 151 μg/mouse

Anti-Mouse CD8 Antibody: 128 μg/mouse

Rat Serum Ig: 132 μg/mouse

The total amounts administered in the case of antibody administration alone were calculated from the absorbed amount. The amounts were the same.

(d) Measurement: Three days after the two administrations, the peripheral blood lymphocytes, the lymph nodes and spleens were removed. The lymphocytes in these organs were stained by the anti-CD4 antibodies and the anti-CD8 antibodies. A two-color FACS analysis was performed to measure the lymphocyte subpopulation percentages.

[0047] (e) Results

Lymphocyte Subpopulation Measurements: See Table 11 through Table 13.

[0048]

[Table 11]

Lymphocyte Subpopulation in Spleen (%)

	CD4+	CD8+	CD4-CD8-	CD4+CD8+
No Treatment	23.98	15.63	59.69	0.70
Rat Serum Ig	26.91	15.98	56.30	0.80
Rat Serum Ig-HA	22.34	13.88	62.77	1.01
Anti-Mouse CD4 Antibody	4.05	17.68	76.86	1.42
Anti-Mouse CD4 Antibody-HA	5.58	15.73	77.78	0.91
Anti-Mouse CD8 Antibody	19.96	0.18	79.38	0.48
Anti-Mouse CD8 Antibody-HA	23.49	0.26	75.94	0.32

[0049]

[Table 12]

Lymphocyte Subpopulation in Lymph Node (%)

	CD4+	CD8+	CD4-CD8-	CD4+CD8+
No Treatment	38.20	26.95	34.02	0.84
Rat Serum Ig	40.09	27.75	31.32	0.85
Rat Serum Ig-HA	38.47	26.91	33.56	1.06
Anti-Mouse CD4 Antibody	4.90	41.58	52.14	1.38
Anti-Mouse CD4 Antibody-HA	8.19	39.40	50.69	1.71
Anti-Mouse CD8 Antibody	47.46	0.20	51.64	0.70
Anti-Mouse CD8 Antibody-HA	48.23	0.37	50.74	0.67

[0050]

[Table 13]

Lymphocyte Subpopulation in Peripheral Blood Lymphocyte (%) /7

	CD4+	CD8+	CD4-CD8-	CD4+CD8+
No Treatment	29.24	15.12	55.25	0.39
Rat Serum Ig	27.77	14.35	57.57	0.31
Rat Serum Ig-HA	29.44	15.09	55.10	0.36
Anti-Mouse CD4 Antibody	2.16	18.01	79.57	0.26
Anti-Mouse CD4 Antibody-HA	4.58	21.35	73.80	0.27
Anti-Mouse CD8 Antibody	25.50	0.23	74.01	0.26
Anti-Mouse CD8 Antibody-HA	31.23	0.17	68.53	0.08

[0051] When the change in lymphocyte subpopulations after administration was observed, it was clear that the antibodies reached the target sites in both the HA-absorbed antibody administered group and the antibody administered group. However, the results from the HA-absorbed antibody administered group were closer to those of normal mice than the antibody administered group. The effect on the body is different depending on whether the different proteins were absorbed by HA and administered or administered in a free state. This reflects more accurate targeting.

[0052] Working Example 10

Because the test results in Working Example 9 suggested the possibility of targeting, a clinical test was performed on experimental animal tumors using targeting. The rat antibodies (anti-mouse IL-2 receptor antibodies) were used in targeting, and NCS was used as the object of therapy. A carcinostatic such as adriamycin can be absorbed by HA alone, but NCS cannot be absorbed by HA alone. A calcium-bonded peptide consisting of a 10-residue amino acid (Asp-Leu-Asp-Glu-Asp-Val-Ser-Gln-Glu-Cys or CBP) was synthesized and the NCS

was bonded to this using the maleimide method to obtain a complex compound able to be absorbed by HA.

[0053] (a) HA-Absorbed Antibody Preparation

The antibodies (final concentration 250 μm/ml) and CBP-NCS (final concentration 50 μg/ml) were added to an HA aqueous suspension (5 mg/ml), and nanomizing was performed three times at 1000 kg/cm^2. After allowing absorption to occur overnight at 4°C, the HA was retrieved using centrifugation and the unabsorbed substance was removed. After HSA (0.5 mg/ml) was added, nanomizing was performed twice at 1000 kg/cm^2 to obtain a sample. The final composition of the sample is shown below.

HA: 2.68 mg/ml

CBP-NCS: 26.8 μg/ml

Rat Antibody: 134 μg/ml

(b) Mice: BALB/c Mice (6 Weeks Old, Female, 5 Mice per Group)

(c) Tumor Cells: BALBRV4 leukemia cells in which an IL-2 receptor had been expressed were ip transplanted (day 0).

(d) Amount Administered and Times Administered: Twice, once on day 1 and once on day 3, ip administration was performed using the amounts shown in Table 14. In the case of NCS alone, three times this amount was administered to another test group.

[0054]

[Table 14]

Group Name	Administered Amount
Group 1	HA 0.27 mg, CBP-NCS 2.7 μg, Ab 13.4 μg/shot
Group 2	CBP-NCS 2.7 μg, Ab 13.4 μg/shot
Group 3	NCS 8.1 μm/shot
Group 4	Physiological Saline Solution

[0055] The results from (e) are shown in FIG 1. Compared to the control group, a life-extending effect was observed in all groups administered the drug. In the group administered a mixture of antibodies and NCS, a life-extending effect of approximately 50% was observed. A very high effect was observed in the groups administered both the HA-absorbed antibodies and the NCS complex compound. Three of five mice were still alive after 50 days, and two of the surviving mice had no observable tumor formation at all. When administered a drug absorbed by the HA carrier as in the present invention or when administered the drug alone, the efficacy of even a small amount was observed in the case of the former. This suggests either that the drug more effectively reached the target site or that the drug had longer-lasting efficacy due to gradual release. Therefore, absorption by the HA carrier of the present invention may suppress side-effects due to administration of more than the efficacious amount of a drug.

[0056]

[Effect of the Invention] Because the HA fine particle carrier in the present invention does not aggregate and has good dispersion stability, it does not have any side effects such as vascular occlusion. Because this carrier absorbs biologically active substances well, it can be used to obtain a highly efficacious and stable /8

medicinal preparation for injection if a biologically active substance is absorbed in the carrier.

[Brief Explanation of the Drawings]

[FIG 1] A graph showing the anti-tumor effect (number of survivors) of the NCS-absorbing HA fine particles.

[FIG 1]